USACEHR TECHNICAL REPORT 0601

ASSESSMENT OF PROTOCOL DESIGNED TO DETECT ENDOCRINE DISRUPTING EFFECTS OF FLUTAMIDE IN *XENOPUS TROPICALIS*

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January 2006

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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
11-01-2006	Technical Report	Jan 2005 to Dec 2005

4. TITLE AND SUBTITLE
ASSESSMENT OF PROTOCOL DESIGNED TO DETECT ENDOCRINE DISRUPTING EFFECTS OF FLUTAMIDE IN XENOPUS TROPICALIS

5a. CONTRACT NUMBER

5b. GRANT NUMBER
EPA IAG DW-21-93962001-0

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
US Army Center for Environmental Health Research
568 Doughten Drive
Fort Detrick, MD 21702-5010

8. PERFORMING ORGANIZATION REPORT NUMBER
Technical Report 0601

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Environmental Protection Agency
OPPTS/OSCP
1200 Pennsylvania Ave, NW (7203)
Washington, DC 20460

10. SPONSOR/MONITOR'S ACRONYM(S)
USEPA

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
This technical report represents a pilot study to test a new protocol with an amphibian frog species, Xenopus tropicalis, for possible application in the U.S. Environmental Protection Agency (USEPA) Endocrine Disruptor Screening and Testing Program. The frogs were exposed to the model anti-androgenic compound flutamide under flow-through conditions for a period of 30 weeks, beginning at 48 hours post-hatch. The endpoints evaluated for this study were (1) overall health of the frogs, (2) body lengths and weights, (3) weights of liver and ovary/egg masses, (4) histopathology of gonads, and (5) plasma vitellogenin levels. Although considerable variability was encountered in the results, statistically significant differences in most endpoints were detected among frogs with different exposure concentrations to flutamide. The authors concluded that development of a X. tropicalis bioassay to screen chemicals for endocrine disruption in amphibians is feasible. Recommendations include : (1) Modification of the test design, (2) Development of additional endpoints, and (3) Careful selection of test chemicals for bioassay development.

15. SUBJECT TERMS
Xenopus tropicalis, amphibian frog bioassay, endocrine disruption, flutamide

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Paul L. Knechtges
U	U	U	UU	45	19b. TELEPHONE NUMBER (Include area code) (301) 619-2332

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Standard Form 298 (Rev. 8/98)
Prescribed by ANSI Std. Z39.18

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Preface

This technical report represents a pilot study to test a new protocol with an amphibian frog species, *Xenopus tropicalis,* for possible application in the U.S. Environmental Protection Agency (USEPA) Endocrine Disruptor Screening and Testing Program. The frogs were exposed to the model anti-androgenic compound flutamide, which was expected to cause intersex reproductive system development and infertility.

Under Congressional mandate, the USEPA is required to develop and implement a comprehensive testing plan that will detect the potential of environmental agents to cause adverse effects on the reproductive and thyroid systems via endocrine system disruption. One of the tests required in the Tier II phase of this plan is an amphibian reproduction test. The U.S. Army Center for Environmental Health Research (USACEHR) received funding through an Interagency Agreement (IAG) to develop an amphibian test protocol using the frog species *Xenopus tropicalis.*

Prior to receiving funding, USACEHR had demonstrated the capability to breed and raise *X. tropicalis* in-house. Research had also been conducted to optimize the feeding regimen necessary for untreated *X. tropicalis* to thrive during development, including the passage through metamorphosis. Additional research included the development of endpoints to assess the impacts of xenobiotic chemicals on reproductive system parameters. This work was done under Animal Use Protocol USACEHR 01-006.

The objectives of the study were to develop a protocol that could be used for a standard U.S. EPA testing procedure in the Endocrine Disruptor Screening and Testing Program and also to evaluate concentration-response effects of flutamide to X. tropicalis.

We are grateful for the assistance of Aaron Crapster, Robert Bishoff, Ronald Miller, John Lewis, Elizabeth Gehman, William van der Schalie, David Jackson, and Wendy Burman for technical support.

This work was funded under Interagency Agreement (IAG) DW-21-93962001-0 between the USEPA and the USACEHR.

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Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals (NRC, 1996) in facilities that are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International.

Summary

The anti-androgenic compound flutamide (CAS # 13311-84-7) was administered to *Xenopus tropicalis* under flow-through conditions for a period of 30 weeks. Sub-groups of frogs were sacrificed at 11 weeks from each tank for observation of gonadal development. The nominal concentrations of flutamide used in the flow-through exposures were 0 (controls), 10, 100, 250, and 500 µg/L. The average measured concentrations of flutamide over the course of the exposure period for each tank were 0 (control), 1 (control), 8, 9, 52, 53, 130, 152, 199, and 220 µg/L. Flutamide concentrations became increasingly difficult to maintain as exposure time progressed, probably because of microbial metabolism/degradation of the flutamide. One of the controls received a short (<4 hours) exposure to flutamide during day 26 from a flutamide stock leak.

Sub-groups of frogs from each tank were sacrificed at 11 weeks for observation of gonadal development. Since the gross morphology and histopathology of these frogs did not reveal any discernable differences between the tanks, only the 30-week results are included in this report.

All remaining frogs were sacrificed at test termination (30 weeks) with blood, gonads, brains, livers, and thyroids collected during necropsy. Endpoints evaluated included: (1) overall health of the frogs (observational comments), (2) body lengths and weights, (3) weights of liver and ovary/egg masses, (4) histopathology of gonads, and (5) plasma vitellogenin levels. The brain and thyroid tissues were either processed for histopathology or frozen in liquid nitrogen and stored at -80°C for possible future analysis.

Overall, the exposure protocol and test design were considered suitable for bioassay development. Mortality in the study animals was negligible and unrelated to the exposure conditions. One hundred percent of the frogs passed through metamorphosis during the predicted time period. Furthermore, the necropsy and tissue preparation procedures used at the terminal sacrifice worked well.

Significant differences in body weights and lengths, as measured by linear regression, were observed among frogs in the flutamide-exposed tanks. Male frogs exhibited a significant increase ($P = 0.008$) in body weight with increasing flutamide concentrations, but differences in lengths of the males were not significant ($P = 0.34$). Among female frogs, the body weights and lengths both decreased significantly ($P = 0.014$ and 0.001, respectively).

The histopathological observations of the male and female gonadal tissue did not reveal any significant differences between the controls and the flutamide-exposed tanks. However, the gross morphological observations of gonads revealed an increasing proportion of frogs with non-recognizable gonadal tissue (NRGT) compared with male frogs and with increasing levels of flutamide concentrations ($P = 0.004$, using logistic regression). Ovary weights decreased with increasing flutamide concentrations, even after adjustment for decreasing female body weights ($P = 0.002$).

As analyzed by linear regression, liver weights increased significantly ($P = 0.042$) among male frogs with increasing flutamide concentrations, even after adjustment for increased male body weights. Liver weights among female

frogs decreased with increasing flutamide concentrations, but these differences were not significant when adjusted for decreasing female body weights (P = 0.54).

The ELISA-based assay for plasma vitellogenin, originally developed for *Xenopus laevis*, also worked well for *X. tropicalis*. Plasma vitellogenin levels differed by several orders of magnitude between male and female *X. tropicalis*. Plasma vitellogenin levels decreased in normal female frogs with increasing flutamide concentrations. This result is not unexpected, because both the liver and ovary weights of females also decreased with increasing flutamide concentrations, and vitellogenin is produced by the liver through induction by intercellular signals from the ovary.

Based on the results of this pilot study, the authors concluded that development of a *X. tropicalis* bioassay to screen chemicals for endocrine disruption in amphibians is feasible. This conclusion, however, is contingent upon further improvements of the bioassay in the following areas:

- Modifications to experimental design.
- Development of additional endpoints.
- Careful selection of test chemicals for bioassay development.

1. Introduction

A substantial body of scientific research suggests that many synthetic chemicals in the environment have the potential to modulate the normal functioning endocrine systems in all orders of vertebrates as well as invertebrates; as a consequence, endocrine disruption has been negatively linked with early development, behavior, and reproduction of biological organisms (Markey et al, 2003). While the U.S. Environmental Protection Agency (USEPA) has some data available on endocrine-disrupting pesticides, sufficient scientific data are not available on most of the estimated 87,000 chemicals currently being produced to allow for an accurate evaluation of endocrine-associated risks.

Since the science related to measuring and evaluating endocrine disruption is relatively new, it is still unclear whether current testing methods have been adequate to detect the potential for these compounds to be endocrine disrupters. It is also unknown what additional testing may be needed to assess and characterize both human health and ecological risks. Validated testing methods are still being developed to determine these risks.

The Food Quality Protection Act of 1996 requires the USEPA to develop and implement a screening program using validated tests for determining the potential in humans for estrogenic effects from pesticides and other man-made compounds. As a consequence of this requirement, the USEPA established an Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to obtain advice on the development of a screening program. The committee groups consisted of stakeholders and technical experts. USEPA proposed a two-tiered approach to gather information needed to identify endocrine disruptors and take appropriate regulatory action, as mandated by Congress. Tier 1 would identify the potential of a substance to interact with the endocrine system, and Tier 2 would confirm this interaction and characterize the effects of the potential endocrine disrupting compound at various concentrations.

One of the Tier 2 tests recommended by the EDSTAC was an amphibian growth, development, and reproduction study. Currently, a validated amphibian test method to address growth, development, and reproduction does not exist. In this report, the U.S. Army Center for Environmental Health Research (USACHER) has utilized the Tropical Clawed frog, *Xenopus tropicalis*, in a partial life-cycle/reproduction test method.

The objectives of the current study were three fold. First, the rearing conditions for untreated frogs were assessed to optimize development under conditions that are necessary to conduct long-term exposure experiments. Parameters evaluated included: 1) the optimization of a feeding regimen and tank water replacement for the different phases of *X. tropicalis* development; 2) new feeding regimens, including a comparison of Nasco frog diet with the University of Virginia's Advanced Tadpole Diet (ATD), and the assessment of other food (frozen brine shrimp and black worms); 3) the examination of the relationship between feeding frequency and developmental rates; 4) the examination of the relationship between tadpole/froglet density and developmental rates; 5) development of a minimally invasive method for the

removal of algal growth in the test tanks; 6) an assessment of the ability of tadpoles to complete metamorphosis when stressed by chemical exposure; 7) an assessment of the growth and survival rates as the animals passed through various stages of development; and 8) an assessment of the time required for animals to reach sexual maturity.

The second objective was to evaluate developmental and reproductive endpoints of *X. tropicalis* that could be used to assess the effects of chemical exposure. These endpoints included: 1) body weights and lengths at time of sacrifice; 2) liver weights at time of sacrifice; 3) gross anatomic and histological characterization of *X. tropicalis* reproductive development; 4) the ability to identify intersex animals from gross necropsy observations as well as from histopathology; 5) determination of histopathological standards for the testis; 6) oocyte staging guidelines and 7) the development of a plasma vitellogenin assay in *X. tropicalis* using an Enzyme-Linked Immunosorbent Assay (ELISA) kit designed for *X. laevis.*

The third objective of the Tier 2 amphibian test was to perform a trial exposure using the anti-androgenic compound flutamide to *X. tropicalis* in a laboratory setting under flow-through conditions for a period of 30 weeks. The chemical flutamide was selected for this preliminary study based on its well-documented anti-androgen activity. Sub-groups of frogs were to be sacrificed at 11 weeks from each tank for observation of gonadal development. The information previously obtained in objectives 1 and 2 of this study were implemented in the design of the flutamide test protocol for objective three. This protocol would then be transferable for further USEPA testing in the Endocrine Disruptor Screening and Testing Program and also used to evaluate the concentration-response effects of flutamide on *X. tropicalis*. The findings of this three part study are presented in this technical report; objectives one and two are incorporated into the Materials and Methods section, and objective three (the flutamide test) is the main focus of the Results section.

2. Methods

This section describes the overall test design, including environmental test conditions, animal husbandry, breeding, and euthanasia of the experimental animals, (*X. tropicalis)*, dilutor operation, procedures used for obtaining specific test endpoints, preparation of the test compound, sampling of exposure tanks and stock solutions for analytical chemistry, description of the ELISA protocol that was used to determine plasma vitellogenin levels, the procedures used for histopathology evaluation of gonadal tissue, and methods used for the statistical evaluation of the test data.

2.1 Animals

2.1.1 Husbandry

The test organism used for this study was the Tropical Clawed frog, *X. tropicalis*, Nigerian strain. The embryos for the study were obtained in-house (see *2.1.2 Breeding*). Embryos (~800 embryos per chamber) were held in 4 L polycarbonate breeding chambers containing one liter of USACEHR-processed laboratory well water (see *2.4 Environmental Conditions*). The embryos were maintained in total darkness in the incubator at a temperature of 25°C (±2°C). After 24 hr of incubation the chambers were examined, dead embryos were removed, and an additional liter of laboratory well water was added to each chamber. At or before Nieuwkoop and Faber (NF) stage 46 (48 hr post-hatch) the embryos were viewed under a dissecting microscope to assess their development. Any abnormal embryos were culled, and normal embryos were randomly transferred, in groups of five, to 5 gallon test tanks until each test tank contained 40 embryos (see *2.3 Test Design*).

Once transferred into the test tanks, the embryos could be fed. At this time, they received 0.4 g of a Sera® Micron/Nasco® (SMN) powder mixture (50/50 w/w) four times a day (3 times a day on weekends). The 0.4 g was suspended in 10 mL of Milli-Q™ water. At post-hatch day 17, the SMN powder mixture was increased to 0.6 g four times a day until day 27, which was when the tadpoles had reached Nieuwkoop and Faber stage 57 and had usable hind legs. At this point, the tadpoles received 0.5 g of Advanced Tadpole Diet (ATD) 2 times a day along with 0.6 g of the SMN powder mixture 4 times a day. The SMN powder was gradually replaced with ATD until ≥99% of the tank organisms had reached Nieuwkoop and Faber stage 66, signifying the end of metamorphosis. At this stage, which was day 41, the ATD completely replaced the SMN powder mixture and the frogs then received ATD 4 times a day and Nasco® pellets 4 times a day. The amount and frequency of these feedings were adjusted throughout the remainder of the 30 week study based on number of frogs remaining, the age of the frogs, and rate of food ingestion.

2.1.2 Breeding

Adult frogs were induced to mate by injecting 200 IU (males) and 250 IU (females) of Chorulon, a human chorionic gonadotropin (hCG) preparation (Provet, Kansas City, MO) into the dorsal lymph sac. Mating pairs were bred in the dark at 24 ± 2°C in laboratory well water. The embryos were de-gelled in 2% L-cysteine and

rinsed three times in laboratory well water, and then placed in breeding chambers as described above.

2.1.3 Euthanasia

For the interim sacrifice, frogs were euthanized by placing the animals in laboratory well water containing 0.2% benzocaine (Sigma Chemical Co., St. Louis, MO). For the final sacrifice, frogs were euthanized by placing the animals in laboratory well water containing 0.2% MS-222 (tricaine methanesulfonate) (Spectrum, New Brunswick, NJ).

2.2 Test Compound (Flutamide)

Flutamide (4'-Nitro-3'-Trifluoromethylisobutyranilide) CAS# 13311-84-7 was obtained from Mediolast (Milano, Italy). Purity was ≥99% as determined by infrared spectroscopy and High Performance Liquid Chromatography (HPLC). Stability testing by USACEHR analytical chemists showed that the compound was stable for one week in laboratory well water maintained at 25°C.

2.2.1 Preparation and Distribution of Stock Solution

Flutamide (0.18 g) was weighed out daily and placed in a 20 L glass container covered with aluminum foil that contained 18 L of either laboratory well water (for the first 6 weeks of the study) or Milli-Q™ water (from week 7 until the end of the study at week 30) to make a 10 mg/L flutamide stock. The stock solution was stirred overnight and then used to replace the current stock the following day. This procedure was carried out on a daily basis so that the stock was never more than 48 hours old. Leftover stock from the previous day was discarded. The flutamide stock was pumped via a peristaltic pump from the stock bottle to the solenoid dilutor system at a rate of ~50 mLs every 5 min for the entire study. The dilutor system distributed the desired concentrations of test compound to the aquaria that contained the test frogs

2.2.2 Sampling of Stock Solution and Test Tanks

For the first 7 weeks of the test, 20 mL samples were taken daily from each of the 10 test tanks containing the frogs, as well as from the flutamide stock bottle for analytical chemistry. Starting with week 4, daily samples were also taken from the delivery tube leading from the stock bottle to the missing chamber of the dilutor. From weeks 8 through 17, samples were still taken daily from the stock bottle, the tube, and the drain, but test tank samples were taken bi-weekly. From week 18 through 30, tank samples were collected on a weekly basis; other samples were still collected daily. Samples for spike recovery were also taken weekly.

2.2.3 Analytical Chemistry

Samples of flutamide were analyzed in triplicate using an Agilent 1100 series HPLC equipped with a diode array detector, temperature controlled auto sampler and Chemstation software (Agilent, Avondale, PA). Initially, a Supelco C-18 25 X 0.46 cm, 5 µm particle size column (Supelco, Bellefonte, PA) was used for the separation. The mobile phase consisted of 55% Acetonitrile: 45% 0.2%

Phosphoric acid in water with a flow rate of 1.5 mL/min. The injection volume was 100 µl and the wavelength used for calibration was 229 nm. The column was maintained at 35°C with the use of a column heater. Samples were analyzed as soon as possible after receipt in the laboratory and placed in a refrigerated autosampler maintained at 4 °C. These conditions were reassessed when it became apparent that flutamide was unstable. The instability and the appearance of an interfering peak not associated with laboratory well water forced a change in the method to provide lower detection limits. A smaller bore column of a similar material was used to increase the sensitivity of the method. The wavelength monitored was also changed to reduce interferences present in flutamide samples. The column chosen for the separation was a Hewlett Packard ODS 10 X 0.21 cm, 5 µm particle size (Agilent, Avondale, PA). The column was maintained at 35°C with the use of a column heater. The mobile phase was 35% Acetonitrile: 65% 0.2% Phosphoric acid in water. The flow rate was 0.5 mL/min, the injection volume was 100 µl and the wavelength used for the calibration and analysis of the sample was 306 nm. The detection limit for flutamide with this method was 5 µg/L.

2.2.4 Waste Handling

All test solutions were drained through a filter apparatus containing activated carbon to minimize waste. Post-filtration chemistry samples were analyzed daily and if flutamide breakthrough occurred, the carbon was replaced. The USACEHR safety officer disposed of all filter waste and hazardous materials at the termination of testing through the Fort Detrick Hazardous Materials Management Office.

2.3 Test Design

The study design utilized four flutamide exposed populations and one unexposed (control) population. There were two replicate tanks per concentration, and 40 frogs per tank. These 10 study tanks were distributed randomly in the water bath. Because it is not possible to determine the sex of the live frogs until near puberty, it was assumed that there was a 50:50 sex ratio in the early tadpoles. Based on two previous range-finding experiments at USACEHR (unpublished data), the following target flutamide concentrations were decided upon, with the duplicate tanks for each concentration (tank numbers in parentheses): 0 (1,2), 10 (3,4), 100 (5,6), 250 (7,8), and 500 (9,10) ppb. The frogs were exposed to the toxicant at or before Nieuwkoop and Faber stage 46, which is considered to be the start of sexual differentiation. The flutamide exposures lasted for 30 weeks, at which time all remaining frogs were sacrificed (see *2.5 Test Endpoints*). An interim sacrifice of 74 frogs took place at 11 weeks post-hatch. Mortality and grossly-apparent morbidity were checked and logged daily. Observation of behavior or toxicity was based on qualitative evaluation of amphibian behavior and times to reach developmental milestones.

2.4 Environmental Conditions

Husbandry and testing took place in the USACEHR mobile laboratory, located outside of Building 568, Fort Detrick, MD. Both tadpoles and frogs were in 15 L of laboratory well water in five-gallon (18.9 L) glass aquaria with the outside bottoms of the aquaria painted white (to enhance visual observations). The turnover of water from flow-through conditions was 5.4 turnovers/tank/24 hours. Laboratory well water used for rearing and testing USACEHR aquatic organisms was formulated and processed on site. Well water pumped from a 550 ft well was run through a carbon filtration system before being mixed at a 1:1 ratio with tap water that had also been carbon filtered and then treated by reverse osmosis. The mixed water was aerated in a holding tank, filtered through a 10 µm particle filter, heated to 25 ± 1 °C, and distributed to the mobile laboratory. This water was distributed at a rate of 300 ml per five min interval from a diluter apparatus to the test aquaria, which were contained in a stainless steel water bath held at a temperature of 25.5 ± 1.2 °C. Water temperature was checked daily in a control tank and was recorded continuously in the same control tank throughout the study with a strip chart and temperature probe. Water quality parameters of pH, dissolved oxygen, and conductivity were measured weekly in each of the tanks. Alkalinity, hardness, and un-ionized ammonia were measured weekly in a control and a high dose tank. USACEHR real time water quality monitoring was used as a water quality control. The test summary of all of these parameters is shown in Table 1.

A light-dark cycle of 12/12 was used with an average light intensity of 322 lux at the tank water surface. Light intensity was measured weekly until lids were placed on the tanks at week 8; after that, no further light measurement were taken. Tanks were cleaned of debris three times per week by siphoning to control the amount of algal growth in the tanks and remove organic waste. Not more than 50% of the tank volume was removed during siphoning.

Table 1. Water Quality Data During Laboratory Tests

Parameter	Temp (°C)	pH	Un-ionized* Ammonia-Nitrogen (mg/L)	Conductivity (mS/cm^2)	Dissolved Oxygen (mg/L)	Alkalinity (mg/L as $CaCO_3$) *	Hardness (mg/L as $CaCO_3$) *
Mean	25.4	7.2	0.039	0.63	3.22	204	190
Range	24.4-26.7	6.3-7.8	0.003-0.171	0.17–1.2	0.01-6.7	38-206	24-304
Number of Observations	28	28	23	27	27	24	24

* data from Tanks 1 & 10 only

2.5 Test Endpoints

2.5.1 Interim Sacrifice

An interim sacrifice was done at week 11. The numbers of frogs taken from each tank were as follows (numbers of frogs in parentheses after tank number): 1(4), 2(7), 3(8), 4(6), 5(5), 6(7), 7(9), 8(0), 9(9), and 10(19). The number of frogs removed was done to adjust the total number of frogs in each tank to 30. Uneven numbers were due to an escape that had occurred, which resulted in tank 9 having a combination of 250 and 500 ppb flutamide frogs. Gross morphology of the frogs was observed and total body length and total body weight were recorded at necropsy. Livers were removed and weighed and samples were put in RNA*later*® for possible future analysis by the USACEHR molecular toxicology laboratory. The gonadal/kidney/adrenal complex was removed and photographed. The entire complex was immediately fixed in Bouin's solution. Tissues were allowed to fix for 24 hr (±6 hr), this was followed by two consecutive 24 hr (±4 hr) rinses in 70% ethanol prior to storage in 10% formalin. The complexes were photographed again after fixation and sent to American Histolabs, Inc. (Gaithersburg, MD), where they were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

2.5.2 Final Sacrifice

Tissues collection (n = 280 frogs) took place over a 4 day period during week 30 of the study. The frogs were necropsied in an assembly-line fashion. A random numbers sheet was generated for the sacrifice order of the frogs. Frogs were anesthetized one at a time in a beaker of 0.2% MS-222 in laboratory well water. The frogs were removed from the beaker while still alive but were no longer responsive to a pinch reflex test. Body weights and lengths (from snout to end of feet) were recorded and the foot was amputated to obtain 3 -15 µl aliquots of blood. The processing of the frog blood is described in detail in *2.5.3 Blood Processing*.

After obtaining blood, the frog was given to a second technician and the head was removed and given to a third technician, who removed the brain. The second technician opened up the abdominal cavity and removed the liver, which was weighed by a fourth technician. A representative section of the liver was removed and fixed for pathology or frozen in liquid nitrogen for subsequent biochemical evaluation. The second technician also removed the gonadal tissue. Ovaries were weighed, photographed and fixed by a fifth technician. Testes and indeterminate kidney/gonad complexes were not weighed, but were given to the second technician who placed them in fixative. Finally, the carcass was given to a sixth technician who removed the thyroid complex. The seventh technician processed the bloods.

The final disposition of the tissues was decided before the start of the sacrifice. All gonadal tissue went into individual color-coded tissue cassettes which were placed in 4 L plastic containers of Bouin's solution and labeled with the tank number. Sixteen frogs each from tanks 1 – 8 had their brains, livers, and thyroids put in individual cassettes (color-coded by organ) that were also placed in containers of Bouin's solution. The remaining frogs from tanks 1 – 8 had their brains, livers, and thyroid

complexes put in 1.5 mL freezer tubes which were snap-frozen in liquid nitrogen and stored in a -80°C freezer for future molecular biology assays (not reported in this paper). All tank 9 and tank 10 tissues went into Bouin's solution because of tank combining that occurred with these frogs in the earlier phase of flutamide exposure. Tissues were fixed in Bouin's from 4 – 7 days. This was followed by two consecutive 24 hr (±4 hr) rinses in 70% ethanol prior to storage in 10% formalin. The testes, indeterminate gonads, and thyroid complexes were once again photographed after fixation in the event that any tissue changes may have taken place due to the fixative. All formalin-fixed tissues were then sent to American Histolabs, Inc. (Gaithersburg, MD), where they were embedded in paraffin.

2.5.3 Blood Processing

Plasma, obtained from control and treated frogs, was stored at -80°C until utilized for subsequent plasma vitellogenin ELISA's. Three tubes (duplicate tubes for the ELISA's and one extra tube) per frog were used for the assay. Buffers for the blood tubes were prepared ahead of time. Sample Dilution Buffer (SDB) was prepared according to the method described by Mitsui, et al (2003). Briefly, the SDB buffer contained phosphate-buffered saline, pH 7.4, with 1% bovine serum albumin, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Tween ® 20, and 0.5% Block Ace (Serotec, NC). Plasma Buffer (PB) contained 50 mM EDTA and 50 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid. Components of both buffers were obtained from Sigma Chemical Co., St. Louis, MO, with the exception of the Block Ace, which was obtained from Serotec, Raleigh, NC.

Prior to necropsy, two labeled microcentrifuge tubes for each frog were prepared by adding 285 µl of SDB to each tube. Tubes were kept on ice. A 600 µl Microtainer® tube (Beckton Dickinson & Co., Franklin Lakes, NJ) with 5 µl of PB added was also labeled for each frog and was used to collect any additional blood that was drawn. This tube was kept at room temperature.

The entire blood collection process did not exceed 2 min for each frog. Two 15 µl aliquots of blood were collected from each frog using a micropipetter. These aliquots of blood were dispensed into the 2 chilled tubes which contained the SDB. A third aliquot of blood was collected using a 250 µl heparinized capillary tube. This blood was dispensed into the plasma collection tubes containing PB; the volume varied according to health and size of frogs, but generally was about 10-20 µl. All tubes were inverted several times for mixing. The microcentrifuge tubes designated for the ELISA's were kept on ice until enough tubes had been collected to do a spin, at which point the tubes were centrifuged at 8000 X G for 10 min at 4°C. The plasma collection tubes containing the remaining blood were centrifuged at room temperature at 10,000-15,000 X G for 2 min. For each tube, the supernatant was removed, dispensed into a cryolabeled screw cap tube, flash frozen in liquid nitrogen, and stored at -80°C.

2.5.4 Vitellogenin ELISA Assays

A *Xenopus* Vitellogenin (VTG) ELISA KIT™ (Japan EnviroChemicals, Ltd.) was used to evaluate the plasma vitellogenin levels in the blood of all of

the study frogs. The range of detection of the kit for plasma VTG was between 0.2 and 50 ng/mL. Plasma from in-house *X. tropicalis* culture frogs was assayed for VTG levels (both male and female) prior to assaying the actual test frogs in order to determine what dilution levels, if any, would be needed for the flutamide plasma samples. From these range-finding observations, it was determined that the male and NRGT plasma samples could be assayed without dilutions, since they were close to the lower detection limits of the kit. Female plasma samples generally needed to be diluted 1:1000 with the sample dilution buffer that was provided with the kit. The 96 well microtiter plates, provided with the kits, accommodated 23 samples per plate. Therefore, 23 plasma samples plus a spike of a male plasma (for calculation of percent recovery), along with an 8-point standard curve, were run for each assay. Spikes were done by adding 40 µl of the 20 ng/mL vitellogenin stock solution to 120 µl of male frog plasma. Percent recovery of the vitellogenin spike was then calculated. The plasma samples for each run were selected randomly. Random samples were repeated throughout the course of the assays in order to test for reproducibility of the kits, as well as reproducibility between the duplicate plasma samples from the same frog. The kit protocol was followed, and the samples were read at 450 nm on a FLUOstar OPTIMA v1.30-0 plate reader (BMG Technologies, Durham, NC).

2.5.5 Histopathological Evaluation of Gonadal Tissue

An important application of the present study was to evaluate the effects of an endocrine disruptor on reproductive organ development, spermatogenesis and oogenesis in toxicological tests at a practical level. Histopathological examinations were limited to use of paraffin-embedded tissues. Paraffin-embedded, hematoxylin and eosin stained tissues were selected to provide descriptions of reproductive organ development, spermatogenesis, and oogenesis that would be directly applicable to the methods used in those tests.

The photographs of the kidney-adrenal-gonadal complexes from the male and indeterminate frogs that had been taken after formalin-fixation were used to determine where the tissue sections should be cut for histology. From two to five 10µm step sections were taken per complex perpendicular to the long axis of the developing gonad. For the females, the ovarian egg mass was oriented in the paraffin to obtain the largest section possible. One representative section was taken at this orientation. All of these reproductive tissue slides were stained with hematoxylin and eosin by American HistoLabs, Inc. (Gaithersburg, MD).

Testicular tissue sections were examined for the presence or absence of recognizable gonadal tissue, seminiferous tubule atrophy, germ cell degeneration, abnormal germ cell development, multinucleate cell formation, unusually large or small seminiferous tubule lumen, spermatocysts content, presence and/or number of testicular oocytes, sloughed germ cell located within the seminiferous tubule lumen, cellular infiltration into the interstitial space, Leydig cell hyperplasia or hypertrophy, increased interstitial fibrous connective tissue. The sections were also examined

for other events typically observed during spermiogenesis (nuclear condensation and elongation) and spermiation (eccentric orientation of the nucleus within the round spermatid, movement of the elongating spermatids to the periphery of the follicle cell, repositioning of the elongated spermatids toward the follicle cell nucleus and release of the follicle cell and elongated spermatid into the tubular lumen).

Ovarian tissue sections were examined for the presence or absence of recognizable gonadal tissue, oocyte degeneration, abnormal germ cell development, presence of developing male germinal cells, differentiation of the animal and vegetal hemispheres, appearance of cortical granules, lipid accumulation in the cytoplasm, presence/absence, appearance and location of nucleoli during oocytes differentiation.

A crude estimation of the volume/density measurements of ovarian components was determined by point counting. These components were pre-vitellogenic oocytes, vitellogenic oocytes (including post-vitellogenic oocytes), intercellular space, and blood vessels. Pre-vitellogenic oocytes are defined as oocytes that contain a relatively small nucleus and no yolk accumulation within the ooplasm. Vitellogenic oocytes are defined as oocytes characterized by the appearance of yolk granules within the ooplasm. Space was defined as the area between germ cells that did not contain any ovarian or vascular tissue. One ovarian tissue cross section per animal from each of the control and treated animals was utilized in this analysis. Tissues were examined under a binocular Axophote Zeiss microscope equipped with a bright field condenser with a 10 X objective and a 10 X eyepiece fitted with a square lattice containing 100 intersections. The number of intersections or hits on pertinent structures over the entire tissue section was counted by a predetermined and systematic movement of sections across the grid without overlap. The volume density (Vv: the volume of the given ovarian component per unit volume of ovarian tissue) was obtained by dividing the sum of points falling on each structure (Pi) by the total number of points over the tissue (Pt). Absolute volume of each of the ovarian components (V) was determined by multiplying its volume density (Vv) by the fresh ovarian weight (Vo): Vv X Vo.

2.5.6 Histopathological Evaluation of Non-Gonadal Tissue

Paraffin embedded livers were oriented to obtain the largest section possible for histological examination. One representative transverse section through each thyroid gland complex was obtained for histological examination. The location of these sections was determined from the post-fixation photographs. Three representative sections were taken from the brains. The goal was to acquire the telencephalon at the level of the frontal organ or pineal gland, the diencephalons at the level of the optic chiasm, and the mesencephalon at the level of the hypothalamus/pituitary. Each section (3 – 5 μm thick) was obtained by sectioning in a rostral to caudal direction. Liver, thyroid-complex and brain sections were all stained with hematoxylin and eosin.

2.5.7 Sexing of Test Frogs

Normal male frogs were defined as having one or both testes present at gross necropsy. The presence of testicular tissue was confirmed by histopathology. Frogs lacking testes at the time of gross necropsy were classified as "indeterminate" or having "non-recognizable gonadal tissue" (NRGT). The absence of gonadal tissue was confirmed by histopathology.

Female frogs were defined as normal if one or both ovaries were present at the time of gross necropsy and the ovaries could be dissected out, weighted and fixed for subsequent histopathology. Abnormal females were defined as having no weighable or removable ovarian tissue at the time of necropsy.

2.6 Statistical Methods

Logistic regression was used to statistically analyze the relationship between flutamide level and the proportion of frogs with NRGT as compared to the total number of non-female frogs (i.e. males plus NRGT). The statistical tests used were logistic regression for an outcome variable with 2 categories, standard normal regression for a continuous outcome variable, and the Kolmogorov-Smirnov goodness-of-fit statistic to check on the validity of the normal distribution assumption for the continuous variable regression. The tests were written in the S-plus programming language (Everitt, 1994).

3. Results and Discussion

3.1 Test Overview

The amphibian *X. tropicalis* proved to be a suitable test model for a chronic aquatic bioassay using dilutors under flow-through exposure conditions. Mortality in the study animals during the 30 week test was negligible and unrelated to the exposure conditions, and 100% of the frogs passed through metamorphosis during the predicted time period. Necropsy of the frogs, organ harvest, and blood collection for plasma vitellogenin analysis at the end of the study all proved to be successful. Although preliminary chemistry studies indicated that flutamide would be a good model compound for the test design, the target flutamide concentrations in the test tanks became difficult to maintain once the test was underway. The possible reasons for this difficulty are discussed in Section 3.2.

The data obtained from the interim sacrifice performed on days 77 and 78 of the study indicated that there were no discernible treatment-related differences in the frogs (i.e. lengths, weights, and histopathology). These data, therefore, are not analyzed or presented in this report.

On day 54 of the study, tanks 8 and 9 overflowed, releasing some of the frogs. Because the source tank for the individual frogs was not known when they were captured, the frogs were simply combined. This combination of frogs was placed back into tank 9, and the labeling of tank 9 became "250 + 500," indicating it contained frogs exposed to nominal concentrations of 250 and/or 500 µg/L.

3.2 Analytical Chemistry Results

Nominal and measured concentrations of flutamide for the 30-week study are shown in Table 2. The target concentration for the flutamide stock solution was 10,000 µg/L. Measured concentrations were variable in the tanks and considerably lower than the nominal levels. Although stability studies of flutamide in USACEHR laboratory well water prior to the start of the study indicated that flutamide was stable, the actual concentrations of the flutamide stock solution began to drop precipitously beginning at day 40 of the study, causing tank concentrations to fall as well (Figure 1). Stock solution concentrations stabilized after deionized water was used to replaced well water as a diluent on day 45, but test tank concentrations continued to fall. Although the exact cause of the drop in flutamide concentrations is not known, a likely explanation is microbial degradation. The system may have been colonized by bacteria that can degrade the flutamide, or existing bacteria may have developed the ability to degrade the flutamide. Because of the low water solubility of flutamide, it was not practical to compensate for flutamide degradation in the test tanks by increasing the turnover rate of the diluter. Another possibility for increasing tank turnover rate and stabilizing flutamide concentrations would have been to use a carrier solvent for flutamide, but since the test was well along when the degradation problem developed, it was not feasible to add an appropriate solvent control.

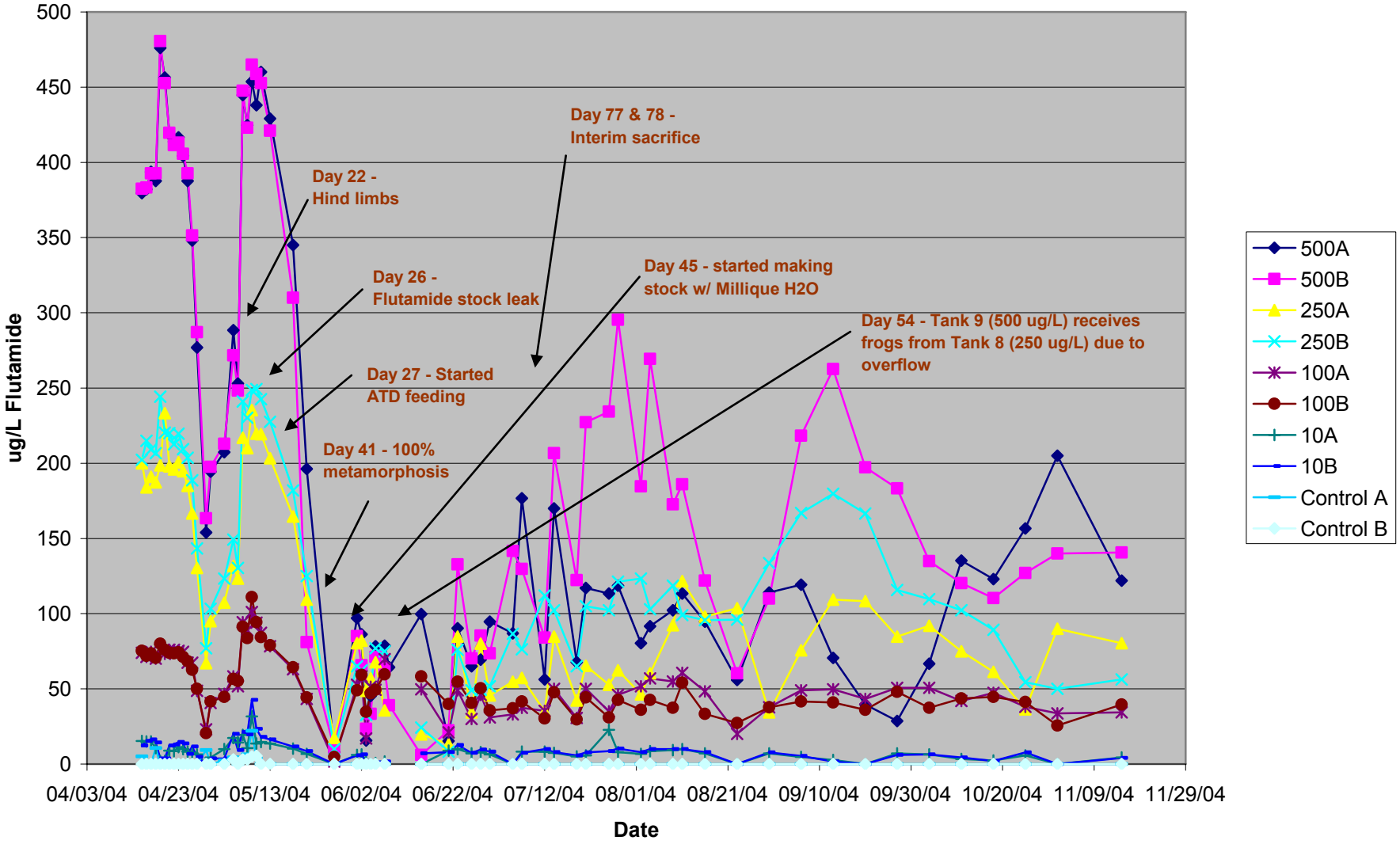
Another issue with flutamide test concentrations occurred because of a leak in the flutamide stock solution tube

Table 2. Summary of Flutamide Chemistry Analysis

Parameter	stock	Tank 1	Tank 2	Tank 3	Tank 4	Tank 5	Tank 6	Tank 7	Tank 8	Tank 9	Tank 10
Nominal (µg/L)	10,000	0	0	10	10	100	100	250	250	250+500*	500
Mean Measured (µg/L)	9,291	1	0	8	9	53	52	152	130	199	220
S.D. (µg/L)	1,796	4	2	6	7	20	20	65	68	150	143
Range (µg/L) (min-max)	0 – 13,392	0 - 27	0 - 9	0 - 34	0 - 44	0 - 105	4 - 115	11 - 240	8 - 252	7 – 506	6 - 484
# of samples	215	65	68	65	65	65	65	65	65	66	66

* Tank 9 had of combination of frogs from tanks 8 and 9 from week 7 until the end of the study as a result of an overflow occurring during week 7.

Figure 1. Measured Flutamide (ug/L) Chemistry Timeline



on day 26, which resulted in control tank # 1 receiving flutamide for approximately 4 hours before the leak was discovered and repaired. The measured flutamide concentration for that day was 22 µg/L. Because of this exposure, frogs in tank #1 were treated as a separate treatment for statistical analysis. Figure 1 illustrates measured flutamide tank concentrations over the study period and significant biological events that occurred.

3.3 Tissue Fixation Method

Immersion fixation of *X. tropicalis* tissues in Bouin's fixative with subsequent dehydration in alcohol and paraffin embedding proved to be an efficient and acceptable method of preserving tissue morphology to permit an accurate histopathological evaluation. Less sectioning was required using this method since large areas of the organs could be surveyed in a single section.

3.4 Testes

3.4.1 Gross Necropsy (Morphology)

Concentration-related effects of the 30-week flutamide exposure were observed for the sexual morphological category "no recognizable gonadal tissue" (NRGT). Table 3 summarizes these effects based on observations made at the time of necropsy as well as subsequent histopathological examination of the gonadal tissue slides. Considering the anti-androgenic effects of flutamide, it was assumed the NRGT frogs were genetic males. Using logistic regression analysis, there was a statistically significant increase ($p = 0.004$) in the proportion of NRGT frogs to the total number of male and NRGT frogs with increasing flutamide concentrations (analysis not shown). Two of the frogs in tank # 2 and one frog in tank # 4 had only one testis, but they were still classified as normal males.

Control tank # 1, which accidentally received flutamide briefly, contained 28.6% NRGT animals (4). In contrast, control tank # 2 had only 5.5% NRGT animals (1). This observation suggests that transient flutamide exposure during critical developmental time points might perturb the development of male gonads. Since only 14 male frogs were involved, the dataset is too small for reliable statistical analysis, and the issue of a critical time period for flutmide exposure cannot be evaluated from the existing study design.

3.4.2 Qualitative Microscopic Analysis

The seminiferous tubules and interstitial compartments of the testes were examined for flutamide-related effects. The arrows in Figure 2A show the seminiferous tubules in a control animal; they are highly convoluted and lined with spermatocysts containing germ cells undergoing spermatogenesis. Each spermatocyst contains a cluster of germ cells at the same spermatogenic phase of development. Differentiated spermatocysts, which line the seminiferous tubule lumen, contain clones of germ cells at different spermatogenic phases of development. All phases of spermatogenesis (spermatocytogenesis, meiosis and spermiogenesis) appeared normal in control and flutamide-treated frogs.

Germ cell degeneration was not observed during the meiotic phase of spermatogenesis in either the control group or any of the flutamide treatment

Table 3. Distribution of Sexes of Frogs in Treatment Tanks								
Tank #	**Flutamide Level (µg/L)**	**Total Frogs**	**Total Normal Males[1]**	**Total NRGT[2]**	**% NRGT (Σ=Males+ NGRT)**	**Total Normal Females[3]**	**Total Abnormal Females[4]**	**% Abnormal Females**
2	**0**	29	17[a]	1	*5.5*	10	1	*9.1*
1*	**1**	30	10	4	*28.6*	13	3	*18.7*
3	**8**	26	14	2	*12.5*	8[c]	2	*20.0*
4	**9**	29	13[b]	4	*23.5*	10	2	*16.7*
6	**52**	30	15	3	*16.7*	11	1	*8.3*
5	**53**	29	17[b]	3	*15.0*	8	1	*11.1*
8	**130**	18	5	5	***50***	7	1	*12.5*
7	**152**	30	11	3	*21.4*	12	4	*25.0*
9**	**199**	30	11	5	*31.2*	10[d]	4	*28.6*
10	**220**	29	5	5	***50***	14[c]	5	*26.3*

[1] "normal males" are defined as having both testes unless otherwise indicated.
[2] "NRGT" = non-recognizable gonadal tissue as indicated by histopathological observations.
[3] " normal females" are defined as having both ovaries present unless otherwise indicated.
[4] "abnormal females" are defined as having no weighable ovaries present at time of necropsy.
* Control tank accidentally exposed to 22 µg/L flutamide for several hours 3 weeks into study.
**This tank had a combination of frogs from tank 8 and 9 from week 7 until the end of the study as a result of an overflow occurring during week 7.

[a] 2 of the frogs in this tank have only one testis.
[b] 1 frog in this tank has only one testis.
[c] 1 frog has only one ovary.
[d] 2 frogs have only one ovary

groups. During normal spermiogenesis, early round spermatids containing condensed nuclei are observed throughout the spermatocyst. As nuclear elongation begins, the spermatid become positioned around the periphery of the spermatocyst. As spermiogenesis continues, the spermatocyst opens and spermatids whose nuclei are becoming more elongate are positioned within crypts formed by the follicle cell. The nuclei of these elongated spermatids are positioned adjacent to the follicle cell nucleus (Figure 2A).

Testicular oocytes, sloughed germ cells lying within the seminiferous tubule lumen, cellular infiltration of the interstitial space, and an increase in interstitial fibrous connective tissue were occasionally observed in treated and untreated frogs, but the effects were not concentration-related. Although the frogs were examined for adverse effects such as seminiferous tubule atrophy, germ cell degeneration, abnormal germ cell development, multinucleate cell formation, seminiferous tubules with unusually large or small lumen, and Leydig cell hyperplasia or hypertrophy, none of these effects were observed. Typical adverse effects (if observed) associated with spermiogenesis would include effects on spermatid nuclear condensation and elongation. Adverse effects associated with spermiation would also include perturbations in the orientation of the spermatid nucleus within the rounded spermatid during early phases of spermiogenesis, abnormal positioning of the elongating spermatid as they become entrenched within the follicle cell, abnormal positioning of the elongated spermatid nuclei with respect to the follicle cell nucleus at later phases of spermiogenesis or failure to release the follicle cell, along with the follicle nucleus and elongated spermatids nuclei into the tubular lumen.

In general, the interstitial cell compartments of both the control and flutamide treated animals were indistinguishable. Leydig cells and occasional collecting ducts were observed in the interstitial space of both the control and flutamide treated animals. These results are summarized in Table 4. Histological examination of the gonadal region of frogs classified as NRGT revealed only the kidneys and the aorta or other large blood vessels and thin sheets of fibrous connective tissue located along the mid-line between each kidney. No evidence of gonadal tissue was observed in these animals (Figure 2B).

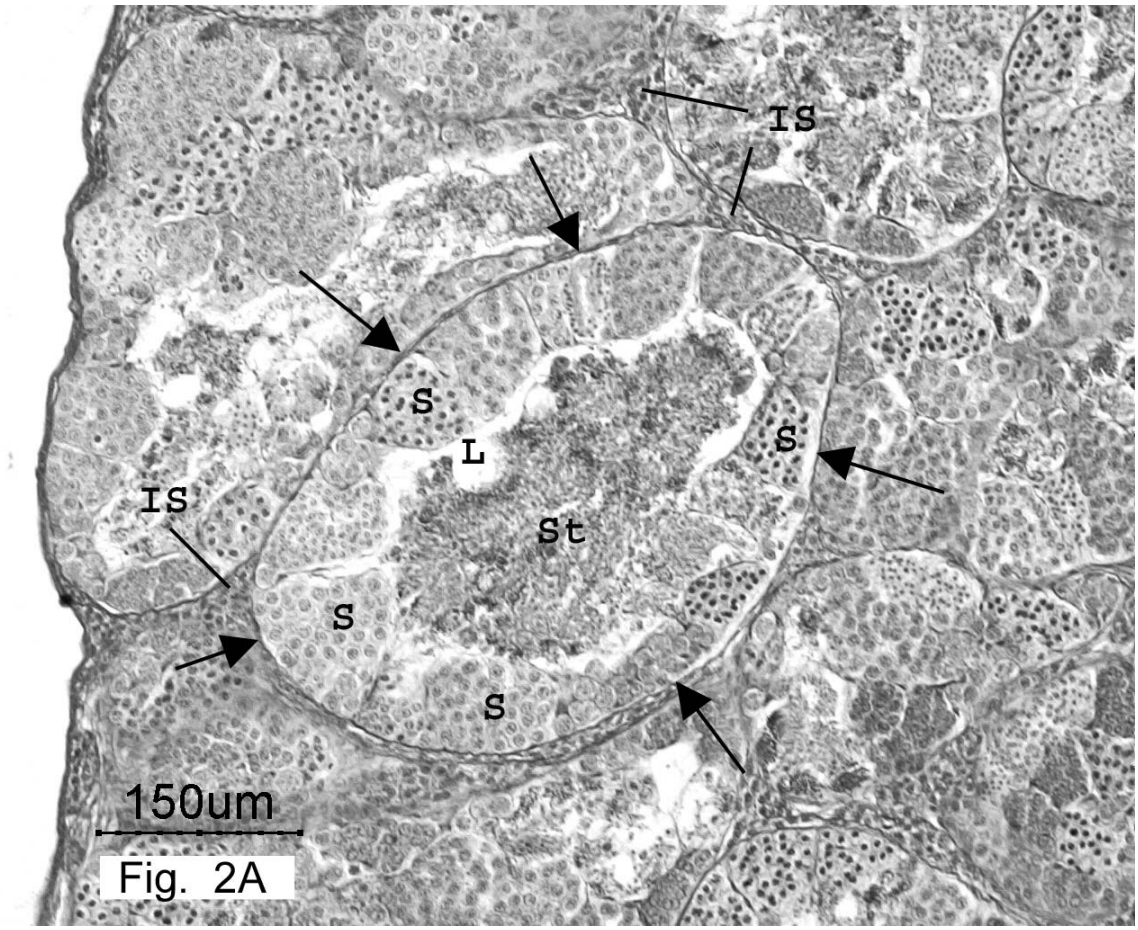


Figure 2A. Photomicrographs of Testes in Control Animal F25

IS = interstitial space, Arrows = boundry of seminiferous tubule,
S = spermatocyst, L = lumen,
St = spermatids released into lumen of seminiferous tubule.

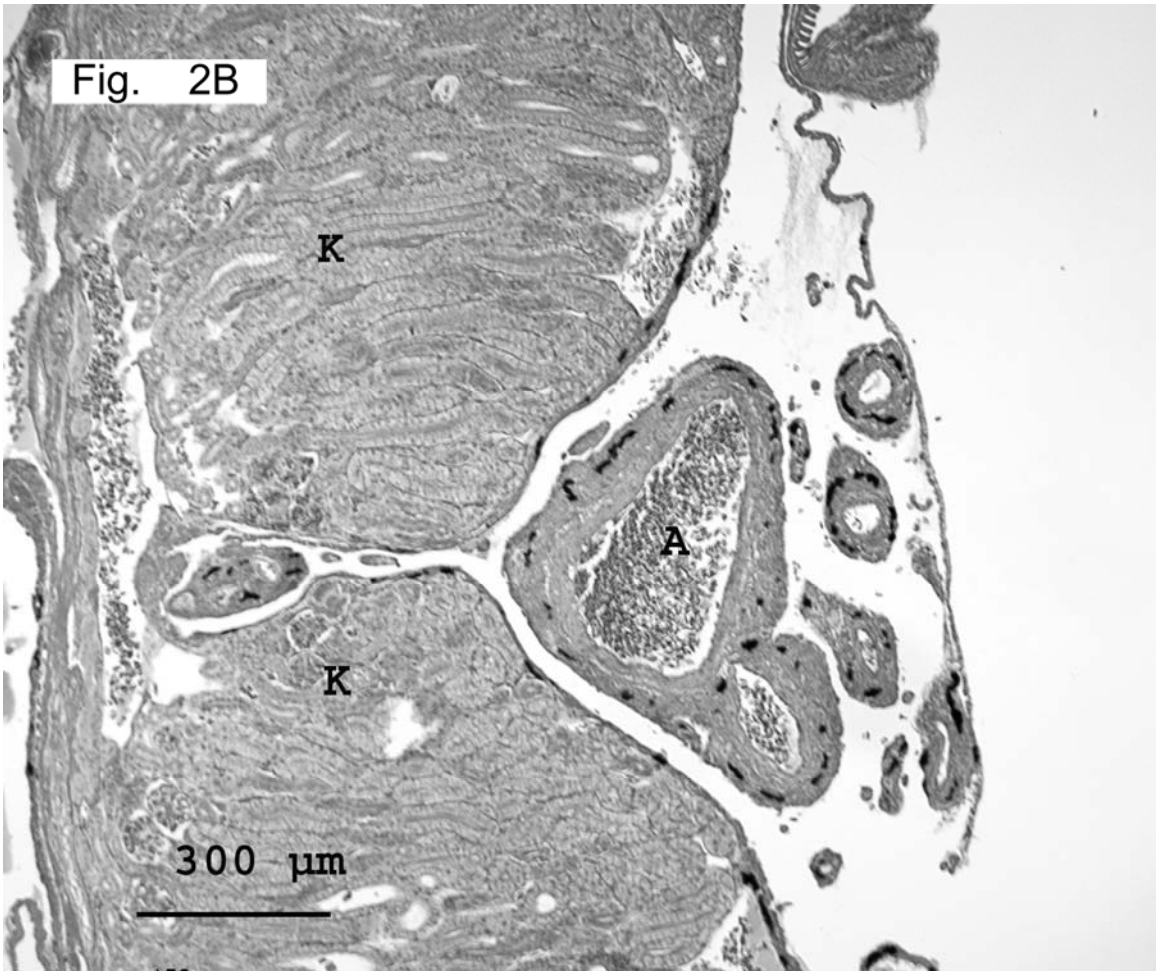


Figure 2B. Photomicrographs of NRGT in Animal F 96

K = kidney, A = Aorta

Table 4. Summary of Male Gonadal and NRGT Tissue Slides Results.										
Tank #	**2**	**1**	**3**	**4**	**6**	**5**	**8**	**7**	**9**	**10**
Flutamide Level (µg/L)	**0**	**1**	**8**	**9**	**52**	**53**	**130**	**152**	**199**	**220**
Total # frogs =	**18**	**14**	**16**	**17**	**18**	**20**	**10**	**14**	**16**	**10**
No Recognizable Gonadal Tissue (NRGT)										
# frogs =	**1**	**4**	**2**	**4**	**3**	**3**	**5**	**3**	**5**	**5**
% of Total	**5.5**	**28.6**	**12.5**	**23.5**	**16.7**	**15**	**50**	**21.4**	**31.2**	**50**
Seminiferous Tubules										
Remaining frogs	17	10	14	13	15	17	5	11	11	5
Normal frogs	16	8	14	12	12	15	4	6	8	5
Degenerating Germ Cells	0	2 F,a	0	0	0	0	0	1 F,b	0	0
Testicular Oocyte Number	2	2	0	0	3	2	0	10	3	0
Testicular Oocyte	1	1	0	0	3	1	0	3	2	0
Sloughed Cells	0	0	0	0	0	0	1 F,a	2 F,a	0	0
Lumen – Large	2 F	0	0	0	0	0	0	1 M,c	0	0
Lumen-Small	0	0	0	1 F	0	0	0	1 M,c	0	0
Many pre-meiotic cysts	0	0	0	0	0	0	0	0	0	0
Reduced/No spermatocyst	0	0	0	1 F	0	0	0	1 F,a	0	0
Female gonadal tissue present with testis	0	0	0	0	0	1	0	0	1	0
Interstitial Space										
Normal frogs	14	7	12	12	14	17	5	10	11	5
Cell Infiltration	2 F,a	2 F,a	1 F,a	1 F,a	0	0	0	0	0	0
Increased Interstitial FCT	1 F,a	1 F,a	1 F,a	0	1 F,a	0	0	1 F,b	0	0

F=Focal; M= Multifocal; a= very mild (few observations); b=mild (observations in 1/3 of tissue); c=moderate (observed in approximately 2/3 of the tissue)

3.5 Ovaries

3.5.1 Gross Necropsy (Morphology)

The percentage of abnormal female frogs (defined as females without removable and weighable ovarian tissue) seemed to increase in each treatment group with increasing flutamide concentrations after 30 weeks, but this increase was not statistically significant ($p = 0.15$). Table 3 summarizes these effects.

The mean ovary weights among the normal female frogs are summarized in Table 5. The mean ovary weight among normal female frogs significantly decreased with increasing flutamide concentrations ($p = 0.001$, linear regression analysis); this effect remained significant after adjusting the ovary weights for the body weights ($p = 0.001$). However, as illustrated by the regression plot in Figure 3, the variability of ovary weights within the control and flutamide-exposed tanks is quite high.

3.5.2 Quantitative Microscopic Analysis

The ovaries of the control and flutamide treated frogs contained oocytes at all stages of development as defined by Dumont (1972). Blood vessels, connective tissue, and variable amounts of space could be found interspersed between the developing oocytes (Figure 4A). An examination of ovarian tissues from a number of flutamide treated animals suggested that these tissues contained more pre-vitellogenic/very early vitellogenic follicles than the control group (Figure 4B). As a consequence of this observation, a quantitative morphometric assessment of the tissue sections was conducted in order to test this conclusion.

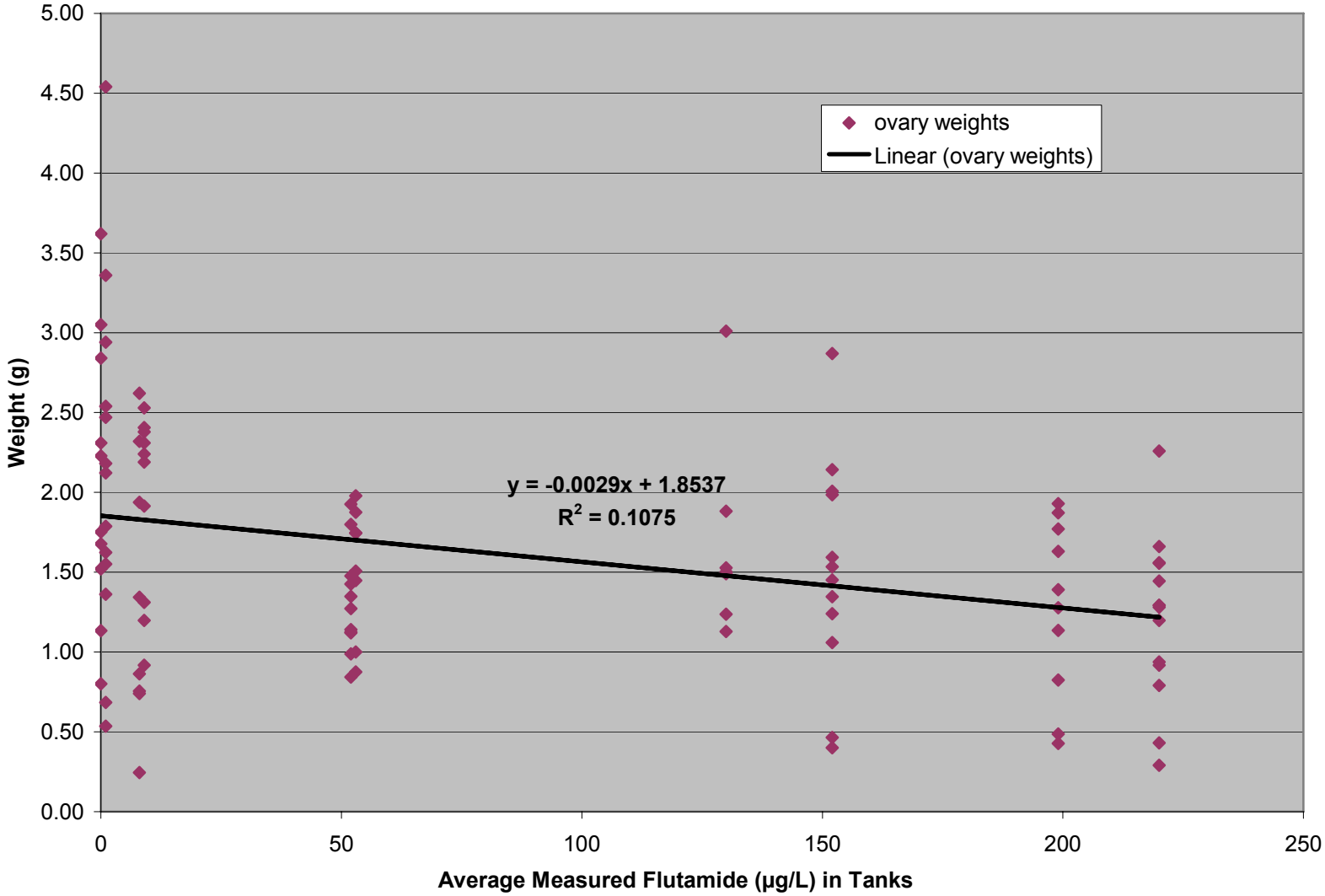
The major findings obtained from the quantitative analysis of the ovarian tissue sections collected from adult female frogs exposed to flutamide are summarized in Table 6. The vitellogenic oocytes occupied approximately 65% and 64% of the ovarian volume density from control animals in tanks 1 and 2, respectively. No trend was observed with increasing flutamide concentrations. The pre-vitellogenic follicles occupied approximately 9% of the ovarian volume density from control animals in both tanks 1 and 2, and the results from the flutamide-exposed tanks were not much different, ranging from 9% to 18% of the ovarian volume density. The volume density of the blood vessels and intercellular space (the area between germ cells that did not contain any ovarian or vascular tissue) was variable.

The absolute volume (expressed in grams) of the vitellogenic oocytes decreased as flutamide concentration increased, but this observation is consistent with the decreasing body and ovarian weights of the flutamide-exposed female frogs (results discussed in *Sections 3.5.2* and *3.8*). The absolute volume of the pre-vitellogenic follicles did not vary from the control values as the flutamide levels increased. No concentration-related response in the absolute volume of the blood vessels was observed over the dose levels utilized in the study. The absolute volume of the intercellular space decreased with the decreasing ovarian weight and increasing flutamide concentrations.

Table 5. Mean ± (S.D.) Ovary Weights (g) for Normal Females		
Tank #	**Flutamide Levels (µg/L)**	**Normal Females[1]** Mean Total Ovary Weight (g) ± (S.D.)
2	**0**	**2.093 (0.887)**
1	**1**	**2.131 (1.088)**
3	**8**	**1.353 (0.852)**
4	**9**	**1.940 (0.581)**
6	**52**	**1.316 (0.329)**
5	**53**	**1.522 (0.401)**
8	**130**	**1.683 (0.632)**
7	**152**	**1.508 (0.698)**
9	**199**	**1.275 (0.551)**
10	**220**	**1.208 (0.512)**

[1] " normal females" are defined as having both ovaries present unless otherwise indicated.

Figure 3. Effects of Flutamide on Ovary Weights in Normal Female Frogs



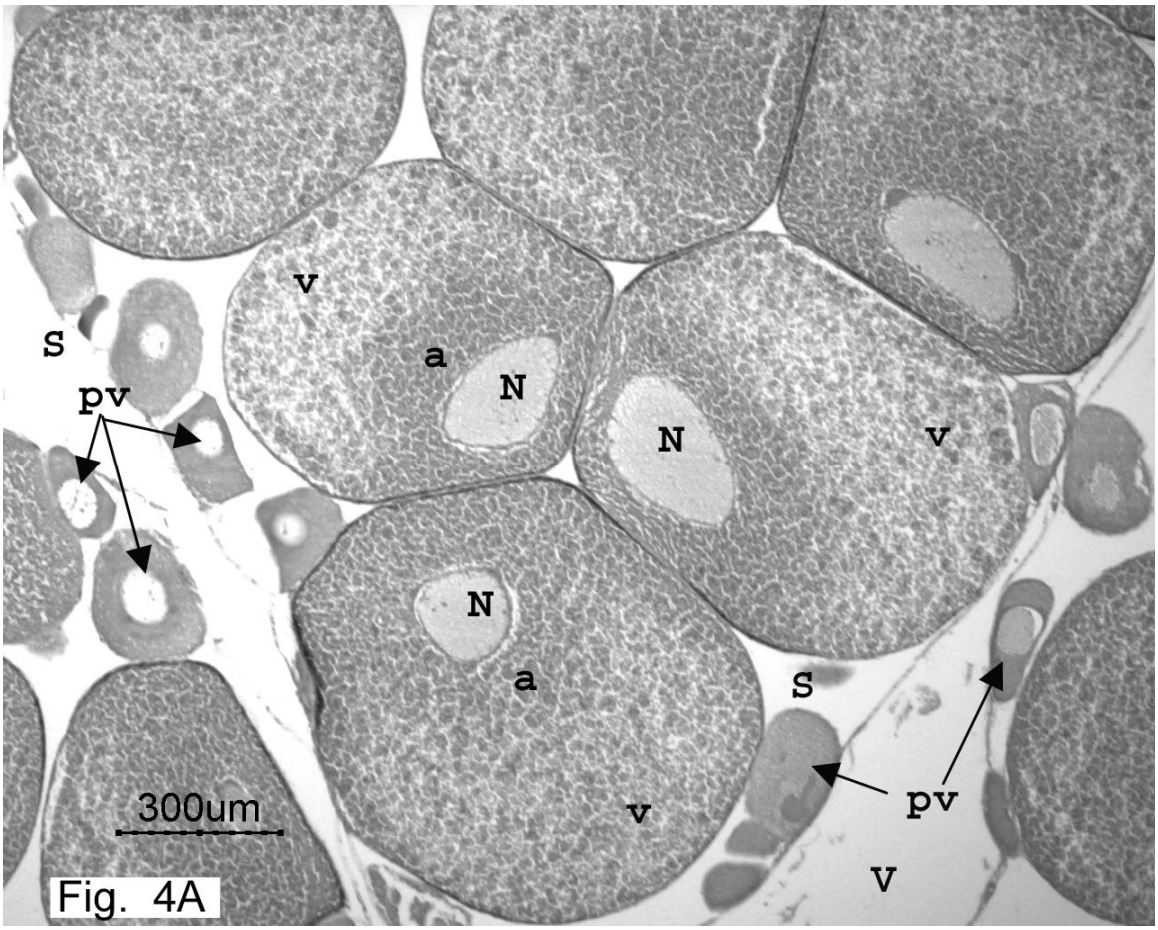


Figure 4A. Photomicrographs of Oocytes in Control Animal 2 - 102

S = space, pv = previtellogenic oocyte, V = blood vessel, n = nucleus, a = animal pole, *v* = vegetal pole.

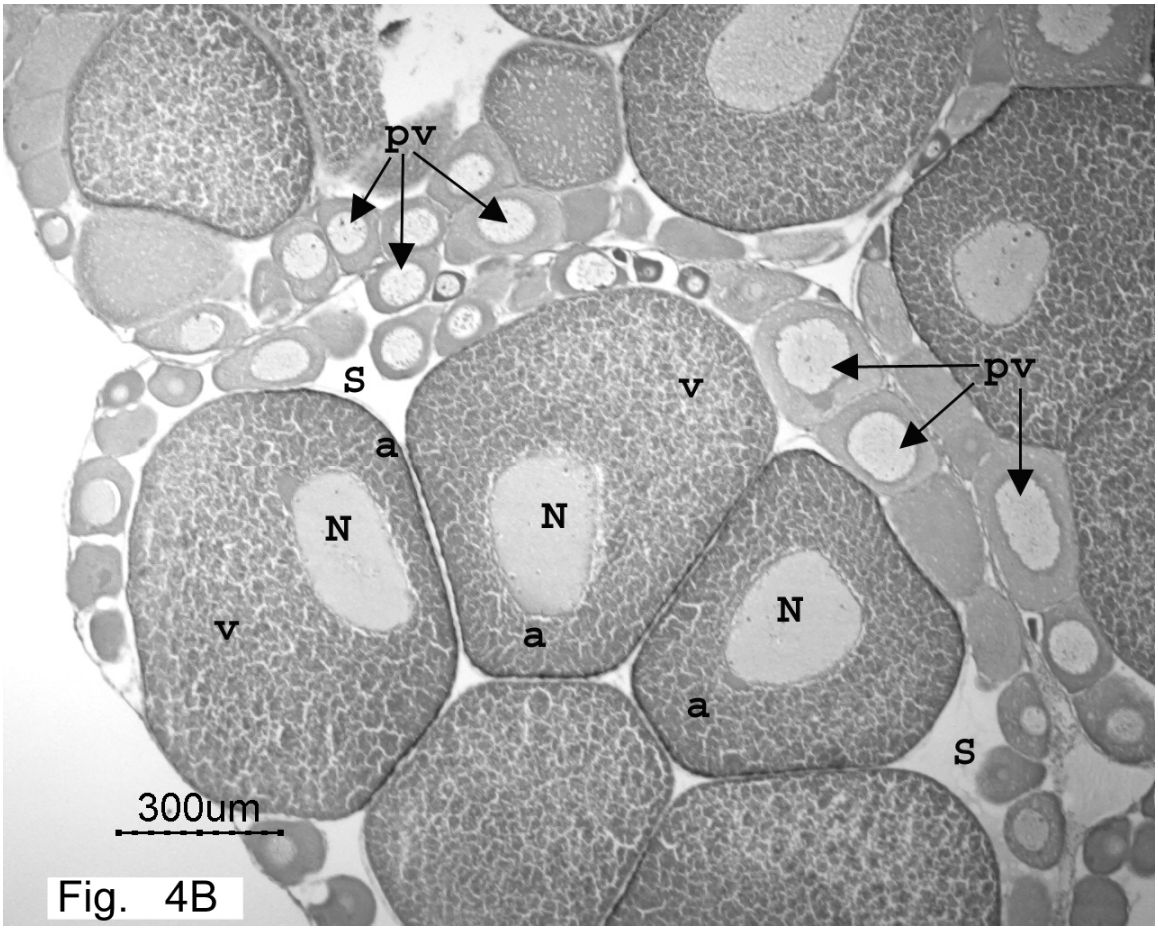


Figure 4B. Photomicrographs of Oocytes in Flutamide-Exposed Animal 10 - 18

S = space, pv = previtellogenic oocyte, V = blood vessel, n = nucleus, a = animal pole, *v* = vegetal pole.

Table 6. Summary of Quantitative Analysis of Ovarian Tissue Sections

		Volume Density*				Absolute Volume**				
Tank #	**Flutamide Levels (µg/L)**	**Vitellogenic Oocytes**	**Pre-Vitellogenic Oocytes**	**Blood Vessels**	**Space**	**Vitellogenic Oocytes**	**Pre-Vitellogenic Oocytes**	**Blood Vessels**	**Space**	**Ovary Wgt (g)**
2	**0**	0.642	0.089	0.003	0.266	0.672	0.089	0.004	0.283	1.047
1	**1**	0.646	0.094	0.026	0.234	0.770	0.078	0.033	0.278	1.159
3	**8**	0.601	0.138	0.000	0.261	0.440	0.072	0.000	0.169	0.680
4	**9**	0.708	0.101	0.008	0.184	0.684	0.095	0.008	0.183	0.970
6	**52**	0.685	0.126	0.022	0.168	0.429	0.073	0.013	0.109	0.624
5	**53**	0.666	0.107	0.009	0.219	0.513	0.079	0.005	0.169	0.766
8	**130**	0.688	0.089	0.011	0.212	0.584	0.072	0.010	0.177	0.842
7	**152**	0.669	0.133	0.015	0.182	0.509	0.082	0.014	0.150	0.754
9	**199**	0.643	0.159	0.011	0.187	0.482	0.093	0.007	0.147	0.729
10	**220**	0.615	0.177	0.004	0.204	0.404	0.086	0.003	0.134	0.626

*The Volume Density is expressed as a ratio of the number of reference points for an ovarian component to the total number of reference points for the entire ovarian tissue. To obtain the results expressed as a percentage of the ovarian volume (Vv %) multiply the volume density (Vv) of each ovarian component by 100.

**The Absolute Volume is determined by multiplying the Volume Density of an ovarian component by the ovarian weight.

3.6 Necropsy Observations of Non-Gonadal Tissue

The mean frog liver weights are summarized in Table 7. Among the normal male frogs the mean liver weights increased significantly with increasing concentrations of flutamide ($p = 0.001$); this trend remained statistically significant after adjusting the liver weight for the body weight ($p = 0.042$). However, no significant relationship between liver weight and flutamide concentration was observed for the NRGT frogs. Among the normal female frogs, a significant decrease in liver weight with increasing flutamide concentrations was observed ($p = 0.008$), but this effect was not observed when the liver weights were adjusted for the body weights ($p = 0.84$). The difference in observations between the liver weights of normal male and female frogs is graphically illustrated in Figure 5 and also shows the high degree of variability within the tanks.

3.7 Histopathology of Non-Gonadal Tissue

No histopathological abnormalities were observed in the livers and brains of the frogs from the flutamide-exposed tanks. Although the sectioning protocol used for the brains and livers provided representative sections of these organs for examination, the sectioning protocol used for the thyroid complex was unsuccessful. No thyroid tissue was found in the expected sections. Further methods development needs to be done on the sectioning of *X. tropicalis* thyroids. At the writing of this report, no post-necropsy work has been done on the frozen tissues.

3.8 Summary of Body Weight and Lengths

The mean body weights and lengths from the study are summarized in Table 8. Among the normal male frogs, a slight increase in mean body weight with increasing flutamide concentration was observed ($p = 0.008$, linear regression), but no trend was detected with mean body length and flutamide level ($p = 0.34$). Among the NRGT frogs, no significant relationships between mean body length or weight and flutamide concentration were observed.

Among the normal female frogs, both the mean body length and weight significantly decreased with increasing flutamide concentration ($p = 0.001$ and 0.009, respectively). However, among the abnormal female frogs, no significant relationships between mean body length or weight and flutamide concentration were observed.

The differences in the observations between the body weights (and their variability) of male and female frogs exposed to increasing levels of flutamide are illustrated in Figure 6.

3.9 ELISA Vitellogenin (VTG) Results

The VTG ELISA kit that was used to measure the plasma VTG levels in the *X. tropicalis* for this study was designed and marketed for *X. laevis*. Methods were developed to adapt the kit for use in *X. tropicalis*, and the results were reproducible. Table 9 is a summary of the average plasma VTG levels for each treatment tank of frogs. VTG levels were 4 – 5 orders of magnitude greater in the normal females as compared to the normal males and NRGT frogs. For the most part, abnormal females had VTG levels that were 1 – 2 orders of magnitude lower than normal females.

Table 7. Means ± (S.D.) of Liver Weights (g)					
Tank #	**Flutamide Level (µg/L)**	**Normal Males[1]**	**NRGT[2]**	**Normal Females[3]**	**Abnormal Females[4]**
2	**0**	**0.223 (0.045)**	**0.388**	**0.349 (0.075)**	**0.232**
1*	**1**	**0.242 (0.070)**	**0.247 (0.023)**	**0.391 (0.086)**	**0.233 (0.056)**
3	**8**	**0.239 (0.042)**	**0.224 (0.117)**	**0.324 (0.064)**	**0.288**
4	**9**	**0.266 (0.119)**	**0.252 (0.102)**	**0.355 (0.068)**	**0.302 (0.071)**
6	**52**	**0.275 (0.039)**	**0.266 (0.091)**	**0.317 (0.048)**	**0.272**
5	**53**	**0.252 (0.051)**	**0.257 (0.032)**	**0.303 (0.059)**	**0.126**
8	**130**	**0.288 (0.063)**	**0.308 (0.060)**	**0.341 (0.061)**	**0.344**
7	**152**	**0.295 (0.044)**	**0.193 (0.080)**	**0.330 (0.091)**	**0.228 (0.084)**
9**	**199**	**0.300 (0.063)**	**0.263 (0.032)**	**0.325 (0.059)**	**0.273 (0.123)**
10	**220**	**0.260 (0.086)**	**0.229 (0.056)**	**0.296 (0.066)**	**0.332 (0.113)**

[1] "normal males" are defined as having both testes unless otherwise indicated.
[2] "NRGT" = non-recognizable gonadal tissue as indicated by histopathological observations.
[3] " normal females" are defined as having both ovaries present unless otherwise indicated.
[4] "abnormal females" are defined as having no weighable ovaries present at time of necropsy.

*Control tank accidentally exposed to up to 22 µg/L flutamide for several hours 3 weeks into study.
**This tank had a combination of frogs from tank 8 and 9 from week 7 until the end of the study as a result of an overflow occurring during week 7.

Figure 5. Effects of Flutamide on Liver Weights in Normal Male and Female Frogs

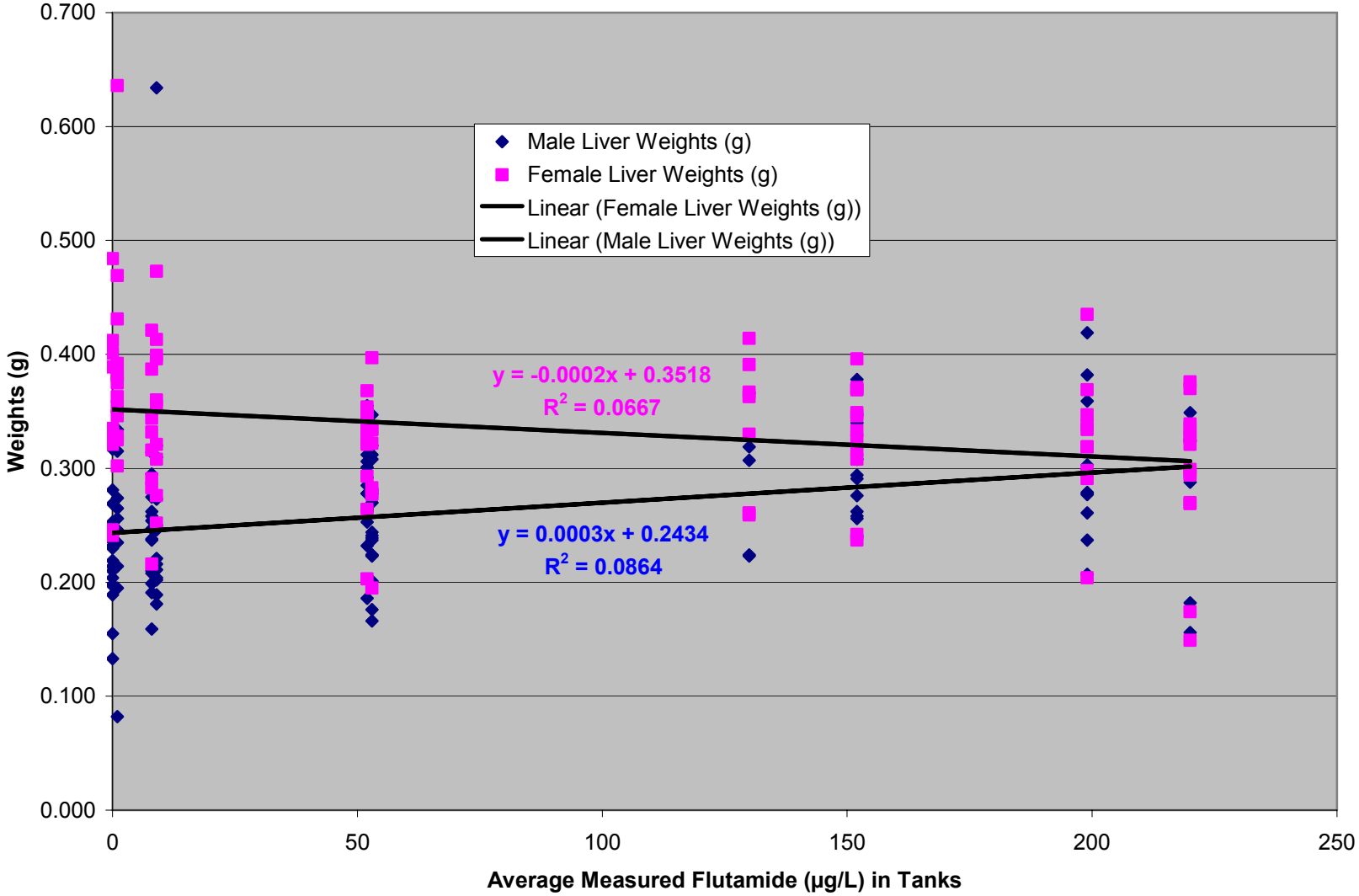


Table 8. Mean ± (S.D.) Body Weights (g) and Lengths (mm)									
Tank #	Flutamide Level (µg/L)	Normal Males[1]		NRGT[2]		Normal Females[3]		Abnormal Females[4]	
		Weights (g)	Lengths (mm)	Weights (g)	Lengths (mm)	Weights (g)	Lengths (mm)	Weights (g)	Lengths (mm)
2	0	9.1 (0.9)	90 (3)	16.6	102	15.2 (3.0)	102 (6)	9.0	94
1*	1	9.5 (1.5)	90 (4)	11.6 (1.7)	92 (2)	16.1 (3.1)	103 (6)	9.7 (1.2)	90 (2)
3	8	9.0 (1.5)	89 (4)	9.5 (3.7)	90 (7)	13.8 (3.0)	100 (5)	11.1 (1.9)	92 (5)
4	9	8.9 (1.2)	89 (4)	11.2 (2.4)	95 (1)	15.2 (3.4)	100 (6)	12.9 (1.5)	105 (9)
6	52	9.5 (1.8)	91 (4)	12.2 (0.9)	99 (1)	12.9 (1.6)	97 (3)	9.5	92
5	53	9.1 (1.6)	89 (5)	10.5 (2.0)	92 (2)	13.3 (1.8)	100 (7)	4.6	72
8	130	10.4 (2.4)	90 (6)	12.8 (1.8)	97 (2)	13.9 (2.9)	97 (5)	11.9	93
7	152	10.4 (1.2)	91 (3)	8.6 (2.1)	90 (6)	13.9 (2.3)	98 (5)	9.3 (2.6)	90 (3)
9**	199	10.4 (1.6)	92 (2)	11.0 (1.5)	93 (6)	13.3 (2.5)	97 (3)	11.1 (3.1)	94 (5)
10	220	8.9 (2.7)	86 (7)	10.3 (3.1)	92 (7)	12.8 (2.9)	95 (4)	12.1 (2.7)	97 (5)

[1] "normal males" are defined as having both testes unless otherwise indicated.
[2] "NRGT" = non-recognizable gonadal tissue as indicated by histopathological observations.
[3] " normal females" are defined as having both ovaries present unless otherwise indicated.
[4] "abnormal females" are defined as having no weighable ovaries present at time of necropsy.

* Control tank accidentally exposed to up to 22 µg/L flutamide for several hours 3 weeks into study.
**This tank had a combination of frogs from tank 8 and 9 from week 7 until the end of the study as a result of an overflow occurring during week 7.

Figure 6. Effects of Flutamide on Body Weights in Normal Male and Female Frogs

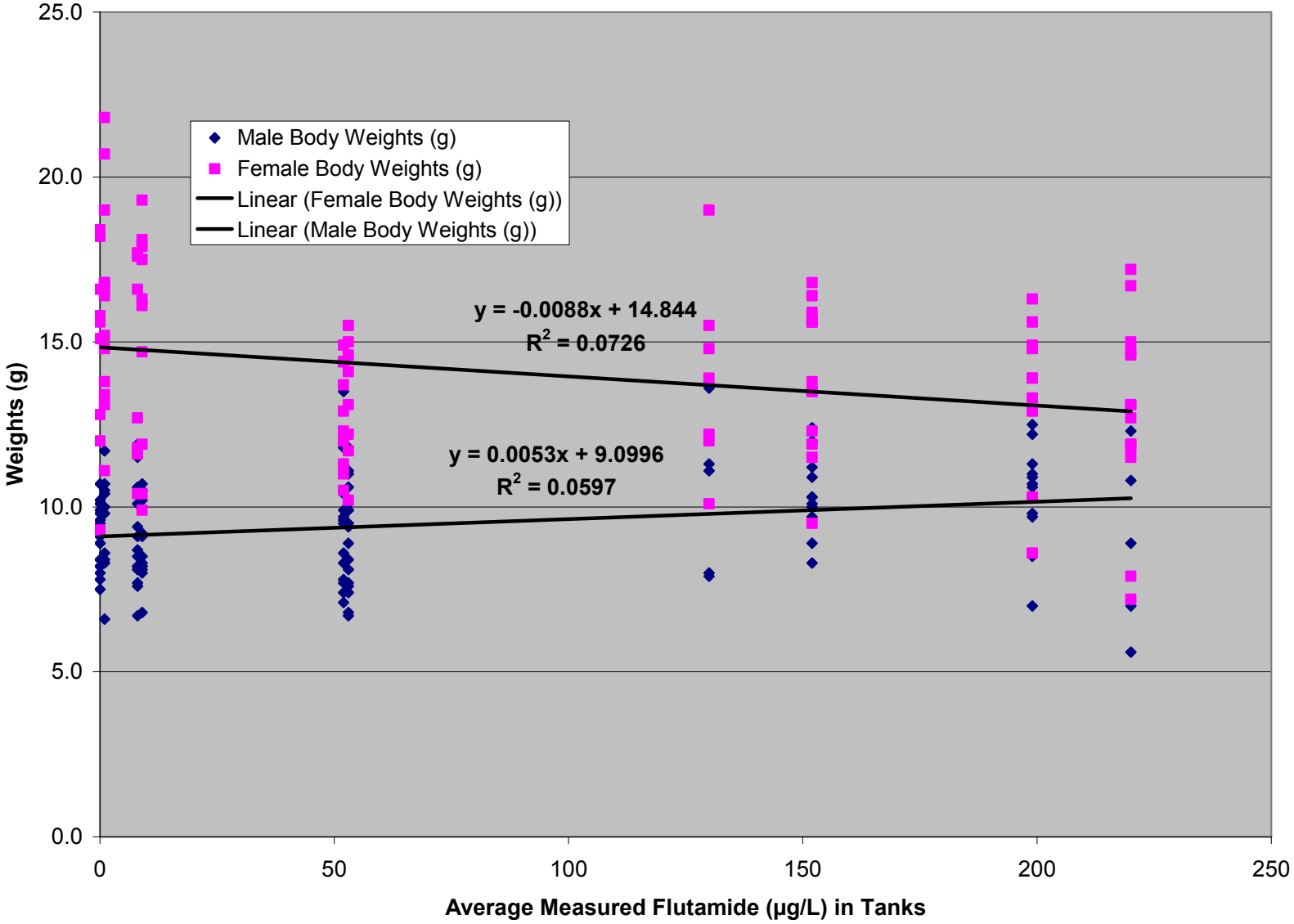


Table 9. Mean ± (S.D.) Plasma Vitellogenin (VTG) Levels (µg/mL)					
Tank #	**FlutamideLevel (µg/L)**	**Normal Males[1]**	**NRGT[2]**	**Normal Females[3]**	**Abnormal Females[4]**
2	**0**	**0.062 (0.136)**	**1.001**	**222 (94.3)**	**0.640**
1*	**1**	**0.025 (0.073)**	**0.379 (0.288)**	**312 (233)**	**26.3 (44.1)**
3	**8**	**0.010 (0.012)**	**0.039 (0.019)**	**171 (69.1)**	**6.13 (7.40)**
4	**9**	**0.012 (0.013)**	**0.020 (0.015)**	**250 (78.4)**	**0.214 (0.056)**
6	**52**	**0.042 (0.073)**	**0.031 (0.026)**	**227 (107)**	**0.893**
5	**53**	**0.100 (0.346)**	**0.233 (0.380)**	**216 (75.8)**	**6.01**
8	**130**	**0.059 (0.084)**	**0.074 (0.093)**	**276 (47.9)**	**28.5**
7	**152**	**0.219 (0.646)**	**0.004 (0.007)**	**254 (53.6)**	**40.8 (75.9)**
9**	**199**	**0.027 (0.025)**	**0.043 (0.055)**	**229 (129)**	**97.3 (159)**
10	**220**	**0.015 (0.009)**	**5.17 (9.89)**	**209 (115)**	**49.2 (99.4)**

[1] "normal males" are defined as having both testes unless otherwise indicated.
[2] "NRGT" = non-recognizable gonadal tissue as indicated by histopathological observations.
[3] " normal females" are defined as having both ovaries present unless otherwise indicated.
[4] "abnormal females" are defined as having no weighable ovaries present at time of necropsy.

*Control tank accidentally exposed to up to 22 µg/L flutamide for several hours 3 weeks into study.
**This tank had a combination of frogs from tank 8 and 9 from week 7 until the end of the study as a result of an overflow occurring during week 7.

Slight but statistically significant increases in VTG levels were found as flutamide levels increased in the combined group of normal males and NRGT frogs ($p = 0.035$). In the normal female frogs, the VTG levels decreased slightly with statistical significance ($p < 0.001$) along with increasing flutamide exposure levels. The observation of decreasing VTG levels with increasing flutamide exposure levels is not unexpected, considering that both the ovary and liver weights of the normal females decreased. (The ovary signals induction of the liver to produce VTG.)

4. Conclusions

Table 10 is a summary of the linear regression p-values for all of the parameters measured and discussed in this report. The table includes p-values for all direct (body length and weight, liver and ovary weight, and plasma VTG level) and weight-adjusted (liver, ovary, plasma vitellogenin) parameters. In summary, as the flutamide level increased, there was a significant increase in body ($p = 0.008$) and liver weight (direct and body weight adjusted, $p = 0.001$ and 0.042, respectively) in normal males. There was also a significant increase in body ($p = 0.039$) and liver weight ($p = 0.022$, direct only) in the combined group of males and NRGT, as well as increased plasma VTG ($p = 0.035$). Normal females had deceased body length ($p < 0.001$), body weight ($p = 0.009$), liver weight ($p = 0.008$), ovary weight ($p = 0.001$), adjusted ovary weight ($p = 0.001$), and decreased plasma VTG ($p < 0.001$) with increasing flutamide levels. Flutamide had no significant effects on any of these measured parameters in abnormal females, but these females by definition had no ovaries. Increasing flutamide levels significantly decreased body lengths ($p = 0.001$), body weights ($p = 0.014$), ovary weights ($p = 0.001$), and adjusted ovary weights ($p = 0.002$) in the combined group of normal and abnormal females.

The great variability in the endpoints measured between exposed groups of frogs in this six-month study may be due to difficulties encountered with maintaining the actual concentrations of flutamide in the flow-through water tanks. The absorbed and target organ doses of flutamide were not determined.

Although this six-month study experienced significant problems related to flutamide concentration stability, this is the first known bioassay attempted with the model species *X. tropicalis* exposed to an endocrine disrupting chemical for six months under flow-through conditions. The frog survival rate was very high using the described protocols, and the sacrifice and necropsy procedures worked remarkably well. With due consideration of all the information and data presented in this report, we believe it is feasible to develop a long-term bioassay using the amphibian *X. tropicalis* to screen potential endocrine disrupting chemicals.

Table 10. Linear Regression P-values for Increasing Flutamide Levels*						
Parameter Analyzed	**Normal Males[1]**	**NRGT[2]**	**All Males (Normal and NRGT)**	**Normal Females[3]**	**Abnormal Females[4]**	**All Females**
Length	0.34	0.42	0.20	<0.001↓	0.48	0.001↓
Body Weight	0.008↑	0.28	0.039↑	0.009↓	0.31	0.014↓
Liver Weight	0.001↑	0.60	0.022↑	0.008↓	0.28	0.054
Liver Weight Adjusted for Body Weight	0.042↑	0.40	0.34	0.84	0.80	0.90
Total Ovary Weight	-	-	-	0.001↓	-	0.001↓
Total Ovary Weight Adjusted for Body Weight	-	-	-	0.001↓	-	0.002↓
Plasma Vitellogenin	0.42	0.16	0.035↑	<0.001↓	0.18	0.33
Plasma Vitellogenin Adjusted for Total Ovary Weight	-	-	-	0.43	-	0.88
Plasma Vitellogenin Adjusted for Total Liver Weight	0.374	0.238	**	0.69	0.25	**

[1] "normal males" are defined as having both testes unless otherwise indicated.
[2] "NRGT" = non-recognizable gonadal tissue as indicated by histopathological observations.
[3] " normal females" are defined as having both ovaries present unless otherwise indicated.
[4] "abnormal females" are defined as having no weighable ovaries present at time of necropsy.

*Significant differences in red. Arrows indicate if difference is due to increase or decrease in parameter as flutamide increases.
** not calculated.

5. Recommendations

5.1 Exposure Conditions and Experimental Design

Based upon the experiences acquired from the study described in this report, the following modifications to the exposure protocol and experimental design are recommended for further bioassay development:

- The issue of chemical stability is generally applicable to any aquatic toxicity study. The problems encountered with maintaining concentrations of flutamide in this study were not identified with preliminary stability testing. Nevertheless, some preliminary testing of chemical stability in water for future bioassays is recommended. It has been suggested that pre-testing chemical stability should be accomplished using *X. tropicalis* under flow-through conditions with tanks containing organic material representative of normal culture conditions. This approach would be costly and time consuming and is not recommended except in special circumstances.
- Although *X. tropicalis* lives totally immersed in water, it is capable of terrestrial locomotion. Escapes by frogs from exposure tanks are possible under the right set of circumstances. Therefore, a means to prevent overflows of tanks and the escape of frogs is recommended foremost. Tanks should be cleaned frequently enough to prevent organic matter from clogging drains and causing overflows, and polyethylene mesh screens should be affixed just above the tops of the exposure tanks (so as to leave an air gap in case the tanks do overflow) to prevent frogs from escaping. Another option is to identify frogs belonging to their respective tanks through some sort of marking system, e.g., toe-clipping. However, unnecessary or disproportionate stress among the tanks of frogs is possible with this approach and is not the best option. Other considerations include colored latex implants, which may be less traumatic.
- The distinction between nominal, free, internal, and target organ concentrations of a test chemical are important, but it is often difficult or impractical to obtain all four concentrations in many aquatic bioassays. In addition, the purpose of the bioassay dictates the need to know particular concentrations (e.g., free concentration for effluent standard setting). For the purposes of developing the *X. tropicalis* endocrine disruption bioassay, knowledge of the nominal and free concentrations is essential. For the development of additional endpoints that measure endocrine disruption in *X. tropicalis*, the determination of internal or target organ concentration is recommended, if possible, to better relate the endpoints to the toxicant exposures. Tissue residues of the

toxicant or its metabolites may be possible to assay, but considerations related to bioavailability, pharmacokinetics, and bioconcentration are important to the frequency and timing of such measurements.

- A desirable but often elusive goal of toxicity testing is to evaluate the risks of exposures at various life stages. For the study in this report, no abnormalities were discernable in the histology of exposed and control frogs taken at the mid-term sacrifice. However, the brief and accidental exposure of one control tank to flutamide resulted in a slightly elevated rate of NRGT frogs, which could not be determined until the tadpoles matured. Partial life cycle exposures at different life stages may be sufficient to cause endocrine disruption, but early endpoints or biomarkers of endocrine disruption have not been validated for *X. tropicalis*. Depending upon the development and relevance of new endpoints or biomarkers, new study designs incorporating partial life cycle exposures and/or early sacrifices should be considered – if feasible – in future studies.

5.2 Additional Endpoint Development

In a recent detailed review paper for an "Amphibian Growth and Reproduction Assay (Tier 2)" by Battelle (2004) for the U.S. EPA, the reviewers discussed a number of noteworthy endpoints: "gonadal development and differentiation and reproductive fitness endpoints, such as egg mass production, measures of ovarian cycle, and general ovary health, sperm count and dysmorphology, and measures of testis health." Some of these measures were used in this study, but improvement in their assessment along with the development of other endpoints is recommended.

- Normal histology baselines for *X. tropicalis* gonads are needed for histopathological endpoint evaluations as are baselines of spermatogenesis for male *X. tropicalis* under flow-through water conditions and for egg mass production in female *X. tropicalis*. A reference atlas or manual is recommended for future evaluations of morphological endpoints.
- The development of additional biomolecular markers of endocrine disruption is recommended. A biomarker for genetic sex determination is particularly important to accurately evaluate sex ratios of frogs exposed to endocrine disrupting chemicals; the critical time periods (if any) for phenotypic sex determination are also important to ascertain. Serum hormone levels and transcriptomic and/or proteomic biomarkers should be investigated for relevance and feasibility. Biomarkers of exposure for target organs may also be helpful. The vitellogenin ELISA-based assay used in this study should be more rigorously validated for use in *X. tropicalis*.
- The multi-disciplinary nature and complexity of endpoint

development require advice from experts. The establishment of an expert advisory group is recommended to assist in the development of additional endpoints.

5.3 Selection of Test Chemicals for Biossay Development

- Compilation and prioritization of known endocrine disrupting chemicals are needed to select future test chemicals for the purposes of bioassay development.
- An expert advisory group should assist in the selection of the next test chemical(s).

6. References

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National Research Council. 1996. Guide for the Care and Use of Laboratory Animals. National Academy Press, Washington, DC.

List of Symbols, Abbreviations, and Acronyms

ATD	Advanced Tadpole Diet
cm	centimeter
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
ELISA	Enzyme-linked Immunosorbent Assay
PB	Plasma Buffer
EDTA	Ethylenediaminetetraacetic acid
hCG	Human chorionic gonadotropin
hr	Hour
IAG	Interagency Agreement
L	liter
mg	milligram
mL	milliliter
min	minute
mS	microSiemens
ng	nanogram
nm	Nanometer
NRGT	Non-recognizable Gonadal Tissue
ppb	Parts per Billion
SMN	Sera®Micro/Nasco®
SDB	Sample Dilution Buffer
µg	microgram
µL	microliter
µm	micrometer
USACEHR	U.S. Army Center for Environmental Health Research
USEPA	U.S. Environmental Protection Agency
VTG	Vitellogenin